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Determination of ML-1035 enantiomers in plasma by chiral high-performance liquid chromatography*

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Abstract: ML-1035, is a gastroprokinetic agent structurally related to metoclopramide. Because ML-1035 contains an asymmetric chiral sulphoxide moiety, a chiral HPLC method has been developed to separate and quantitate its *R*- and *S*-enantiomers in plasma. The ML-1035 enantiomers present in plasma are extracted with dichloroethane under alkaline conditions, the extract evaporated to dryness and reconstituted in the mobile phase. Samples are chromatographed on a Chiralcel OD HPLC column with hexane–absolute ethanol (1% TEA) (1:1, v/v) as the mobile phase. The enantiomers of the unchanged drug are determined by fluorescence measurement (ex: 310 nm, em: 350 nm). The method provides a linear response for both enantiomers over a concentration range of 25 (limit of determination) to 2500 ng ml^{-1} with correlation coefficients of 0.9987 or greater. The inter-assay precision is 9.5% or less and the accuracy ranges from 93.9 to 103.4% of the theoretical value. The method is used to determine the plasma concentrations of the *R*- and *S*-enantiomers following oral and intravenous administration of *R*- or *S*-enantiomers to dogs. The method is also adapted to measure enantiomer levels from *in vitro* reaction mixtures so that the possibility of metabolic inversion may be assessed. The data suggest that no significant level of inversion between the enantiomers occurred either *in vivo* or *in vitro*.

Keywords: *Chiral; enantiomer; ML-1035 pharmacokinetics; HPLC; fluorescence; metoclopramide analogue; sulphoxide.*

Introduction

ML-1035 is a gastroprokinetic agent which is structurally similar to metoclopramide, a widely used anti-emetic and stimulant of upper gut motility [1–3]. ML-1035 is a racemic mixture with its chiral centre in the sulphoxide moiety (Fig. 1). Therefore, the objectives of this study were to develop a sensitive and selective chiral chromatographic method for determining the *R*- and *S*-enantiomers of ML-1035 in plasma, and then to determine the pharmacokinetic profile of the enantiomers following oral and intravenous administration to dogs. Further, the possible inversion of the *R*- and *S*-enantiomers also was investigated both *in vitro* and *in vivo*.

Experimental

Materials and reagents

HPLC grade methanol, hexane, and dichloroethane were purchased from Burdick and Jackson (Muskegon, MI). Ethyl alcohol (absolute) was purchased from Quantum Chemicals Corporation (Tuscin, IL). Triethylamine was purchased from Aldrich Chemical Company (Milwaukee, WI). BMY-25795 (Fig. 1), obtained from the Bristol-Meyers Squibb Company, was used as the internal standard.

Preparation of standard solutions

Stock solutions of the *R*- and *S*-enantiomers and the internal standard BMY-25795 were prepared by weighing 10 mg of each into separate 10 ml volumetric flasks and making up to volume with methanol. Serial dilutions of the stock solution were made in methanol to obtain working standard solutions of 0.01, 0.001 and 0.0001 mg ml^{-1}. Similarly, the internal standard working standards were prepared by diluting the stock solution to 0.01 mg ml^{-1} in methanol. All standards were stored at between 0 and 5°C.

Extraction of plasma samples and calibration standards

The plasma calibration standards were prepared by spiking 1 ml of control dog plasma with the *R*- and *S*-enantiomers at concentration

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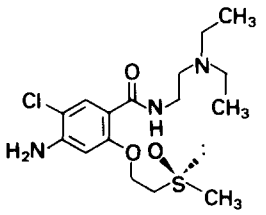
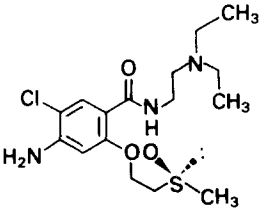
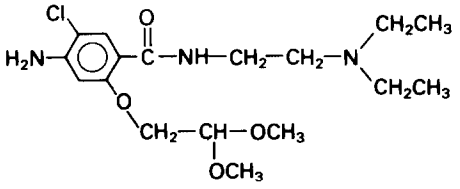


ML-1035 (+)-S-4-amino-5-chloro-2-(2-(methylsulphinyl)ethoxy)-N-(2-(diethylamino)ethyl) benzamide



ML-1035 (−)-R-4-amino-5-chloro-2-(2-(methylsulphinyl) ethoxy)-N-(2-(diethylamino)ethyl) benzamide



Internal Standard

Figure 1
Absolute configuration of ML-1035 and the structure of internal standard BMY-25795.

levels of 0, 25, 50, 100, 250, 500, 1000 and 2500 ng in 15 ml screw-capped glass tubes. The internal standard, BMY-25795, was added at 500 ng by pipetting 50 µl of 0.01 mg ml^{-1} standard solution to plasma samples and calibration standards.

The extraction of the *R*- and *S*-enantiomers from plasma was initiated following the addition of 0.5 ml of 0.2 M Na_2CO_3 saturated with $(NH_4)_2 HPO_4$ and 7 ml of dichloroethane. Tubes containing samples and calibration standards were slowly shaken for 30 min on a mechanical shaker and centrifuged at 3000 rpm for 15 min. The top aqueous layer was aspirated to waste and the dichloroethane layer was evaporated to dryness in a Savant SpeedVac Concentrator (Farmingdale, NY). The dried samples and standards were reconstituted in 0.25 ml of the hexane:ethanol (1:1, v/v) mobile phase. The reconstituted samples were transferred to HPLC vials and a 200 µl aliquot was injected into an HPLC system.

Chromatographic equipment and conditions

The chiral HPLC analysis was performed on a Perkin–Elmer modular liquid chromatographic system (Perkin–Elmer Corporation, Norwalk, CT) equipped with a LC-410 quaternary pump, ISS-100 autoinjector, and a LS-4 fluorescence detector. The detector was set to 310 nm excitation and 350 nm emission wavelengths. A Beckman PeakPro data system was used to collect, integrate and analyse the chromatographic data.

The chromatographic resolution of ML-1035 enantiomers was achieved on a cellulose carbamate bond silica gel column (5 micron Chiralcel OD, 4.6 mm × 250 mm from Diacel USA Inc., Fort Lee, NJ), which is a Type II chiral stationary phase (CSP) [4]. In the past, Type II CSPs have been successfully used to resolve chiral sulphur moieties [5]. The chiral recognition appears to be based on hydrogen bonding, π–π interactions and dipole stacking in the formation of diastereomeric complexes between the analyte and the chiral stationary phase [4].

The mobile phase was prepared by mixing 10 ml of triethylamine and 990 ml of absolute ethanol. This ethanol solution was mixed online with *n*-hexane to form a ratio of 1:1 (v/v) at a flow rate of 0.7 ml min^{-1}. Both mobile phase solutions were degassed with helium prior to use.

In vivo *and* in vitro *studies*

The utility of the method was determined by analysing samples from *in vivo* and *in vitro* studies designed to measure the pharmacokinetic profile and possible metabolic inversion.

The *R*- and *S*-enantiomers of ML-1035 were administered to different dogs (n = 4) by a single iv dose of 2.5 mg kg^{-1} or an oral dose of

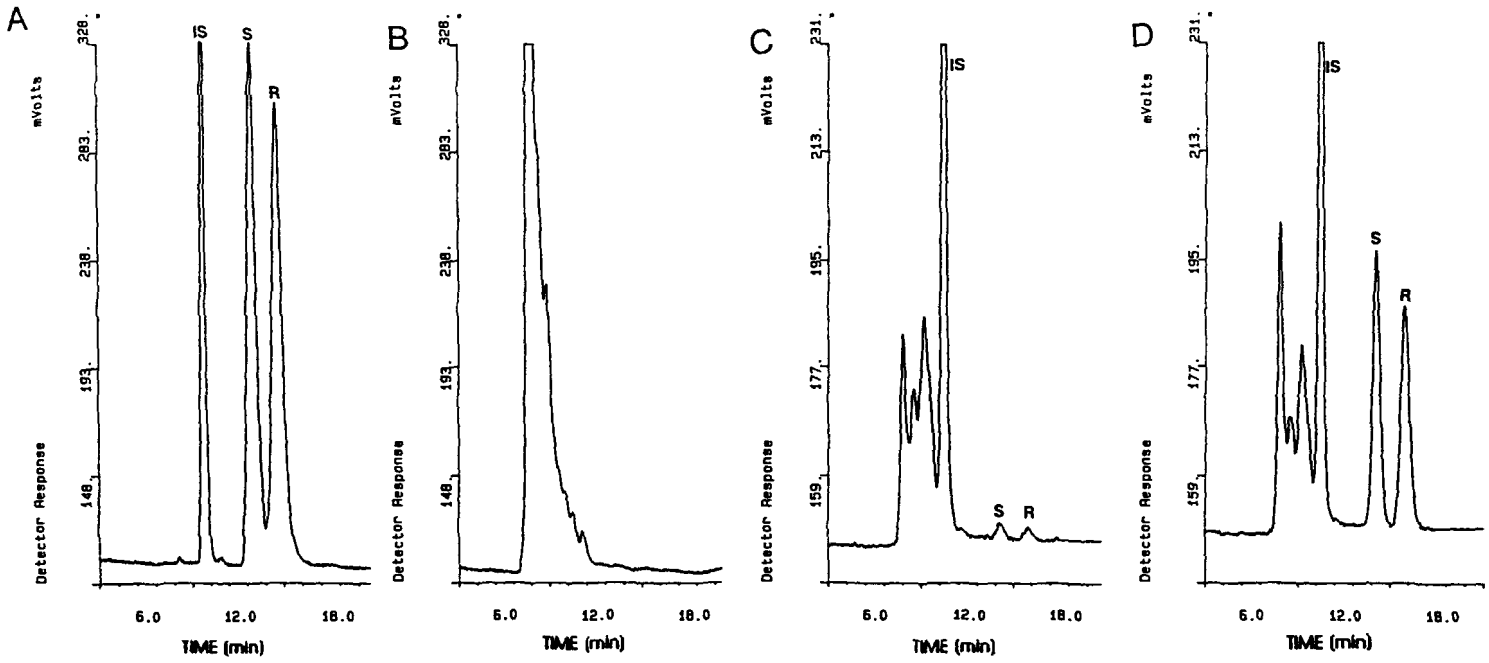


Figure 2
Chromatograms of (A) ML-1035 *R*- and *S*-standard in mobile phase, (B) plasma blank, (C) 25 ng ml^{-1} and (D) 500 ng ml^{-1} of ML-1035 *R*- and *S*-enantiomer spiked dog plasma. Experimental conditions are given in the text.

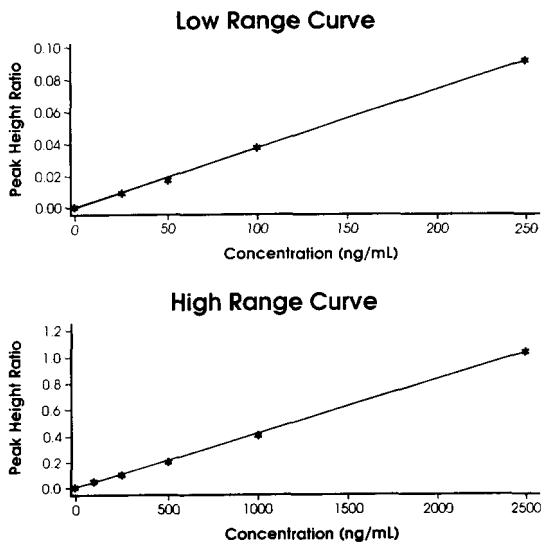


Figure 3
Calibration curves for *R*-enantiomer in dog plasma.

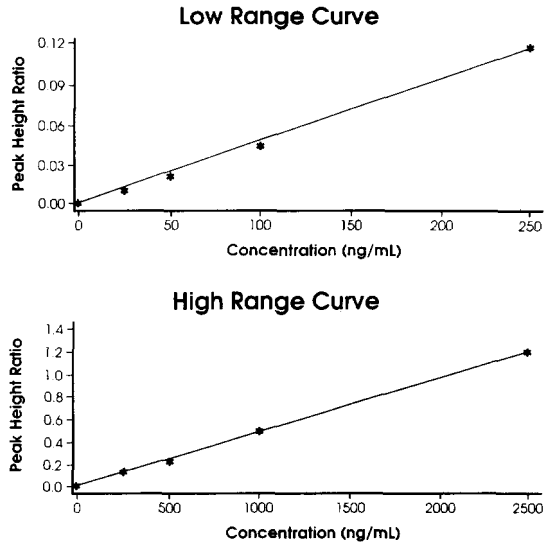


Figure 4
Calibration curves for *S*-enantiomer in dog plasma.

Table 1
Inter-assay* precision and accuracy of the *R*-enantiomer in dog plasma

Predicted concentration (ng ml^{-1})	Number of replicates†	Observed concentration‡ (ng ml^{-1}) mean ± SD	RSD (%)	Relative recovery (%)
0	3	0	—	—
25	3	25.63 ± 1.53	6.0	102.5
50	3	47.99 ± 1.59	3.3	96.0
100	3	103.4 ± 9.8	9.5	103.4
250	3	249.0 ± 3.8	1.5	99.6
500	3	484.3 ± 30.6	6.3	96.9
1000	3	964.1 ± 36.3	3.8	96.4
2500	3	2523 ± 15.3	0.6	100.9

*r values ranged from 0.9987 to 1.000, mean value of slope — 0.0004.
†Three sets of calibration curves containing duplicate standards were run on separate days. The calibration standards were divided into low range curve (25–250 ng ml^{-1}) and high range curve (250–2500 ng ml^{-1}).
‡The observed concentration data for 0–250 ng ml^{-1} was obtained from low range curve and, for 500–2500 ng ml^{-1} data from high range curve.

Table 2
Inter-assay* precision and accuracy of the *S*-enantiomer in dog plasma

Predicted concentration (ng ml^{-1})	Number of replicates†	Observed concentration‡ (ng ml^{-1}) mean ± SD	RSD (%)	Relative recovery (%)
0	3	0	—	—
25	3	25.81 ± 1.82	7.1	103.2
50	3	48.10 ± 3.75	7.8	96.2
100	3	95.99 ± 0.89	0.9	96.0
250	3	251.9 ± 0.83	0.3	100.8
500	3	496.6 ± 25.7	5.2	93.9
1000	3	995.9 ± 73.5	7.4	99.6
2500	3	2508 ± 26.6	1.1	100.3

*r values ranged from 0.9987 to 0.9998, mean value of slope — 0.0005.
†Three sets of calibration curves containing duplicate standards were run on separate days. The calibration standards were divided into low range curve (25–250 ng ml^{-1}) and high range curve (250–2500 ng ml^{-1}).
‡The observed concentration data for 0–250 ng ml^{-1} was obtained from low range curve and, for 500–2500 ng ml^{-1} data from high range curve.

20 mg kg^{-1}. Blood samples were collected at 0, 0.083 (iv only), 0.25, 0.5, 1, 2, 4, 6, 8, 10, 24 and 48 h postdose. Plasma was separated by centrifugation and the samples were stored in a −20°C freezer until analysis.

In vitro, the *R*- and *S*-enantiomers were incubated separately with liver 10 000*g* supernatant (10S) from rat and dog. The incubate contained 0.1 mM of *R*- or *S*-enantiomer, rat or dog 10S equivalent to about 0.6 g of liver, NADPH (1 mM) and an NADPH regenerating system. The reactants were incubated at 37°C for 1 h. The samples were extracted and analysed by HPLC for the *R*- and *S*-enantiomer concentrations.

Results and Discussion

Specificity and linearity

Typical chromatograms of blank and spiked dog plasma in Fig. 2 show that *R*- and *S*-enantiomers were well resolved. The approximate retention time of the *S*-enantiomer was 10.2 min whilst that of *R* was 11.7 min. Further, no endogenous compounds appear to interfere with their determination.

The peak height ratios of the *R*- and *S*-enantiomers (relative to internal standard) were linear over the concentration range of 25–2500 ng ml^{-1}. The calibration standards were divided into a low (25 to 250 ng ml^{-1}) and a high (100 to 2500 ng ml^{-1}) concentration range in order to obtain a more precise fit of the linear regression lines (Figs 3 and 4). Correlation coefficients (r values) of all calibration curves were 0.9987 or greater (n = 3). The limits of quantitation for both enantiomers, measured as the free base, was 25 ng ml^{-1} when 1 ml of plasma was extracted.

Precision and accuracy

The inter-assay precision of the method, as measured by the per cent relative standard deviations at all concentration levels for the two enantiomers on three separate days, was less than 9.5% (see Tables 1 and 2). Similarly, the accuracy of the method, as measured by the per cent relative recovery on three separate

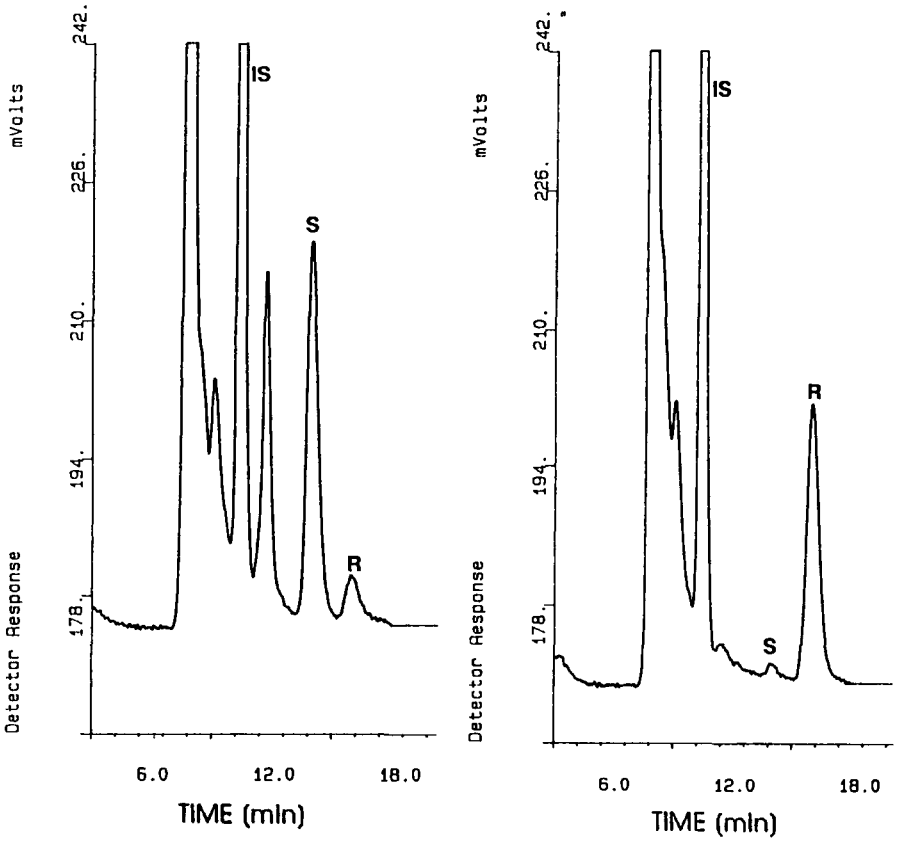


Figure 5
Chromatograms of plasma samples obtained 1 h after iv administration of 2.5 mg kg^{-1} of the '*S*' (left panel) or '*R*' (right panel) ML-1035 enantiomers to dogs. Experimental conditions are given in the text.

days, ranged from 93.9 to 103.4% (Tables 1 and 2).

Method application to in vitro *and* in vivo *samples*

Plasma samples obtained from dogs dosed with the *R*- or the *S*-enantiomer by intravenous or oral route were analysed for the presence of the two enantiomers (Fig. 5). The assay results show that the *R*- and *S*-enantiomer plasma profiles were not significantly different following iv administration (Fig. 6). Similarly, the plasma profiles of the two enantiomers were not significantly different following oral administration (Fig. 7). Data from this study also indicate that no appreciable levels of inversion occurred following oral or iv administration of either the *R*- or the *S*-enantiomer. The results from the *in vitro* liver incubation study also show that the inversion between the two enantiomers was not significant (Table 3).

Conclusions

A chiral HPLC method has been developed and validated for the determination of *R*- and *S*-enantiomers of ML-1035 in plasma. The method is sensitive, selective and reproducible, with a minimum quantifiable concentration of 25 ng ml^{-1} for both *R*- and *S*-enantiomers. The utility of the method was established by analysing plasma samples from dogs treated with *R*- or *S*-enantiomers and no significant differences between the plasma disposition profiles of the samples were found. *In vitro* and *in vivo* study results showed no significant degree of inversion between the *R*- and *S*-enantiomers.

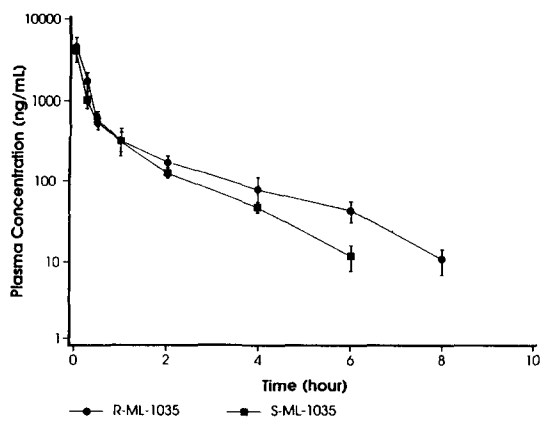


Figure 6
Plasma concentration of *R*- and *S*-ML-1035 in dogs after iv dosing of each enantiomer at 2.5 mg kg^{-1}.

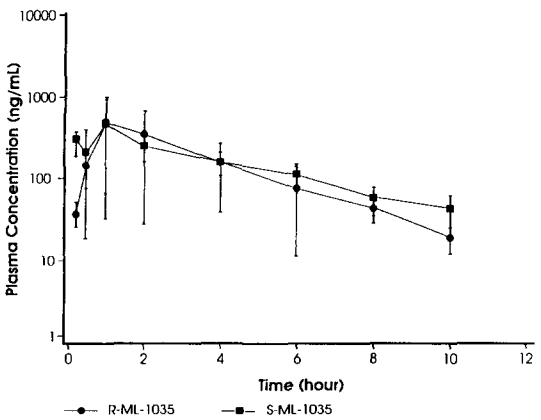


Figure 7
Plasma concentration of *R*- and *S*-ML-1035 in dogs after oral dosing of each enantiomer at 20 mg kg^{-1}.

Table 3
Stereochemical outcome of *in vitro* incubation of *R*- or *S*-enantiomers of ML-1035 in rat and dog liver 10,000*g* supernatant

		Drug concentration ($\mu g\ ml^{-1}$)				
		Initial		Final		
Species	Substrate stereochemistry	*S*-	*R*-	*S*-	*R*-	% Inversion*
Rat	*S*	32.4 ± 3.6	0.2 ± 0.3	15.6 ± 1.1	2.68 ± 0.7	7.7
	R	1.6 ± 0.3	32.5 ± 2.3	0.4 ± 0.2	31.2 ± 2.8	NA
Dog	*S*	40.8 ± 2.5	—	34.7 ± 2.1	2.5 ± 0.9	6.1
	R	1.5 ± 0.2	39.0 ± 2.4	1.0 ± 0.3	37.24 ± 0.5	NA

* Per cent inversion of $R\text{–}S = \frac{\text{Final } S \text{ Conc.} - \text{Initial } S \text{ Conc.}}{\text{Initial } R \text{ Conc.}} \times 100$

Per cent inversion of $S\text{–}R = \frac{\text{Final } R \text{ Conc.} - \text{Initial } R \text{ Conc.}}{\text{Initial } S \text{ Conc.}} \times 100.$

NA — not applicable.

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